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TITLE: Central Pain Mechanisms and Novel Therapeutic Strategies in a Model of Closed Head Injury

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13. SUPPLEMENTARY NOTES					
14. ABSTRACT Headache is the most common, persistent symptom of post-concussion syndrome and highly prevalent following traumatic brain injury of all severities. Inflammation is an early promoter of pain, and is proposed to play an important role in the pathogenesis of chronic post-traumatic headache; however, this role is not well defined. This research investigates the contribution of acute and chronic inflammation to the development of headache after closed head injury. The specific aim (1) was to determine the pattern of inflammation-induced sensitization of the central trigeminal pain neurons, and if sensitization is detectable by quantitative EEG. Sprague Dawley rats were randomized to mild closed head injury (CHI), repetitive mild CHI (rCHI) with two injuries (rCHI2) and three injuries (rCHI3) groups or served as an incision control group to determine the effects of graded inflammatory on central trigeminal pain neurons at acute 1 day and 1 week and chronic 4 week endpoints. Quantitative EEG headache behavioral testing, as well as immunohistochemical and molecular studies uncover underlying inflammatory contributors to post-traumatic headache. An in vitro slice assay was used to test anti-inflammatory and anti-nociceptive mechanisms using a cannabinoid receptor type-2 agonist in trigeminal pain pathway tissues.					
15. SUBJECT TERMS Post-traumatic headache; chronic migraine					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 19	19a. NAME OF RESPONSIBLE PERSON USAMRMC
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- **INTRODUCTION:**

Headache is the most common, persistent symptom of post-concussion syndrome and highly prevalent following traumatic brain injury of all severities. Inflammation is an early promoter of pain, and is proposed to play an important role in the pathogenesis of chronic post-traumatic headache[1]; however, this role is not well defined. This research investigates the contribution of acute and chronic inflammation to the development of headache after closed head injury. We have made substantial progress in determining the pattern of inflammation-induced sensitization of the central trigeminal pain neurons, and if sensitization is detectable by quantitative EEG. Over the last year, we have made considerable progress in characterizing EEG recordings and electrical signatures in a model of repetitive mild closed head injury (rCHI) used to study post-traumatic headache. We have identified at least three unique parameters that identify rCHI animals, and that will serve as excellent endpoint measures that can be used to benchmark and test novel therapeutic strategies in the coming project period. Second, we have made significant progress in our assessments of key anti-inflammatory pathway that inhibit chronic sensitization of central trigeminal pain neurons in a model of post-traumatic headache.

- **KEYWORDS:**

Post-traumatic headache

Post-traumatic migraine

Chronic migraine

Traumatic brain injury

Quantitative EEG (QEEG)

Analgesia

Endocannabinoid

Cannabinoid receptors

Cannabinoid type 2 receptor

- **ACCOMPLISHMENTS:**

▪ **What were the major goals of the project?**

Specific Aim 1 was to determine the pattern of inflammation-induced sensitization of the central trigeminal pain neurons, and if sensitization is detectable by quantitative EEG.

Specific Aim 2: To determine if anti-inflammatory mechanisms inhibit chronic sensitization of central trigeminal pain neurons in vitro

Table 1: Estimated completion dates Year 2		
Major Task	New Estimated Projected completion Date	% Completed
1: Aim 1 Major Task 1: Conduct quantitative EEG testing in a pre-clinical model of concussion	8/14/16	100%
2: Aim 1 Major Task 2: Conduct behavioral testing in rats (<i>Chronic groups</i>)	10/30/16	100%
3: Aim 1 Major Task 3: Completion of post-mortem histology and molecular studies	2/30/16	95%
4: Aim 2 Major Task 1: Conduct In vitro post-mortem brain slice experiments		
Subtask 4a: Perform closed head injury or incision surgery in In Vitro for Acute groups	5/30/2016	100%
Subtask 4b: Perform post-mortem brain slice experiments for Acute groups	10/30/2016	100%
Subtask 4a: Perform closed head injury or incision surgery in In Vitro for Chronic groups	3/1/2017	40%
Subtask 4a: Perform post-mortem brain slice experiments for Chronic groups	3/1/2017	25%

▪ **What was accomplished under these goals?**

1) **Major Activities** for the second year

- Completed all experimental cohorts of chronic closed head injury groups for EEG studies and qEEG analysis. On our current EEG system, *only 4 rats can be run at a time for a 4-week period.*
- Completed chronic behavioral testing.
- Confirmed sources of inflammation and neuronal sensitizers in a model of post-traumatic headache
- Completed analysis of acute cohorts for injury and control groups for slice experiments and conducted chronic cohorts for slice experiments

2) **Study Objectives:**

Aim 1: To determine the pattern of inflammation-induced sensitization of central trigeminal pain neurons, and if sensitization is detectable by quantitative EEG.

Aim 2: To determine if anti-inflammatory mechanisms inhibit chronic sensitization of central trigeminal pain neurons in vitro

3) **Results and Conclusions:**

Sprague Dawley rats were randomized to mild closed head injury (CHI), repetitive mild CHI (rCHI) with two injuries (rCHI2) and three injuries (rCHI3) groups or served as an incision control group to determine the effects of graded inflammatory on central trigeminal pain neurons. Acute endpoints were at 1 day and 1 week, and 4 weeks. Quantitative electroencephalography, headache behavioral testing, as well as immunohistochemical and molecular studies were used to uncover the underlying inflammatory contributions to post-traumatic headache. In vitro slice assay was used to test an anti-inflammatory and anti-nociceptive mechanisms using a cannabinoid receptor type-2 agonist in trigeminal nucleus caudalis tissues. We have completed generating all acute groups and chronic qEEG groups and will be finishing up the remaining chronic slice groups and histological analysis.

Inflammation after mild CHI sensitizes the trigeminal pain pathway in which neurons within TNC are proposed to signal the modulation of pain after injury. Increases in CGRP in capsaicin-stimulated trigeminal nucleus caudalis (TNC) slices are found for rCHI rats but not for incision controls (**Fig. 1**). Notably, our findings show the CB₂R agonist, JWH-133, blocked the capsaicin-induced increases in CGRP and prostaglandin in TNC and forebrain/cerebrum slices (**Fig. 1**). Repetitive CHI induced increases in capsaicin-triggered PGE₂ release in the forebrain/cerebrum slices, but not in the TNC slices (**Fig. 2**) indicating other pain mediators may

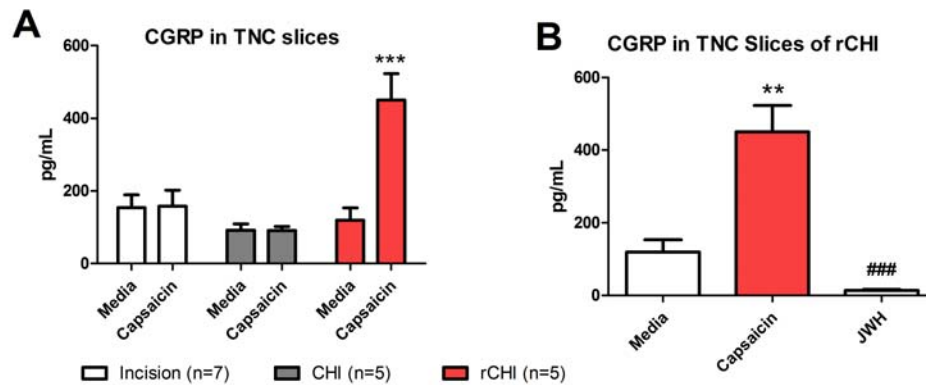


Figure 1: TNC brain slices from incision and repetitive closed head injured (rCHI) rats incubated with media or capsaicin, *** $p < 0.001$ (A). TNC slices incubated in media, capsaicin, or capsaicin + JWH-133 (100 nM), ### $p < 0.001$ compared to capsaicin and ** $p < 0.01$ compared to incision control. (B)

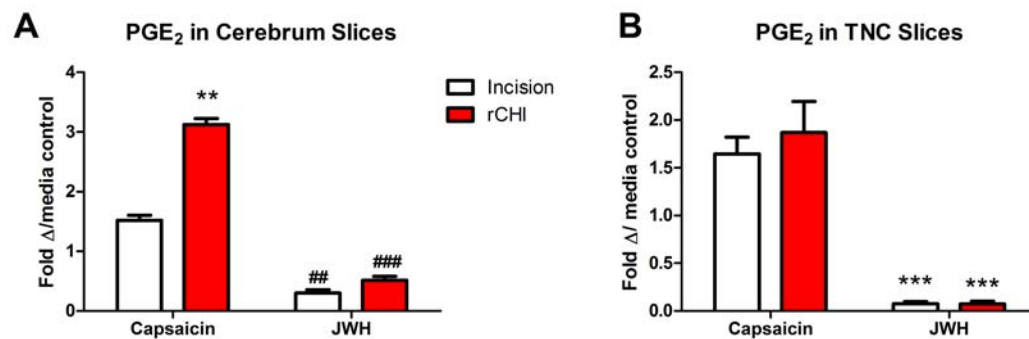


Figure 2: Forebrain/cerebrum TNC and brain slices from incision and repetitive closed head injured (rCHI) rats (Fold change over media control) incubated with capsaicin or capsaicin + JWH-133 (100 nM), ** $p < 0.01$ vs. incision, ## $p < 0.01$, ### $p < 0.001$ vs. capsaicin (A) and *** $p < 0.001$ vs. capsaicin (B).

be important in this region. In a previous study we showed that JWH-133 inhibits TNF- α and iNOS in mice with cortical injury[2]; The role of the CB₂R in inflammation after TBI is evident as mice lacking the receptor show a significant increase in TNF- α mRNA after cortical injury[2]. The increase in TNF- α in mice lacking the CB₂R receptor indicates the importance of the receptor in controlling the inflammatory response; this is supported by studies by our laboratory[3, 4] and others[5-10].⁵⁴. CB₂R are upregulated in reactive microglial cells in neuropathic pain and neuroinflammatory conditions [11, 12]. Chronic single CHI (4 week endpoint) were not different from controls, $p = 0.3$ ($n = 3$ and 5); however we are conducting experiments to determine if changes can be triggered and blocked in tissues with rCHI. Our data demonstrates the importance of this assay to testing experimental compounds in the future as well as mechanisms underlying sensitization in the TNC. Increases in CGRP levels after repetitive CHI persist from day one to week one endpoints after the last injury, whereas single mCHI did not, ANOVA group $p < 0.0001$ ($F = 46.43$) and time $p < 0.01$ ($F = 8.3$) (**Figure 3**).

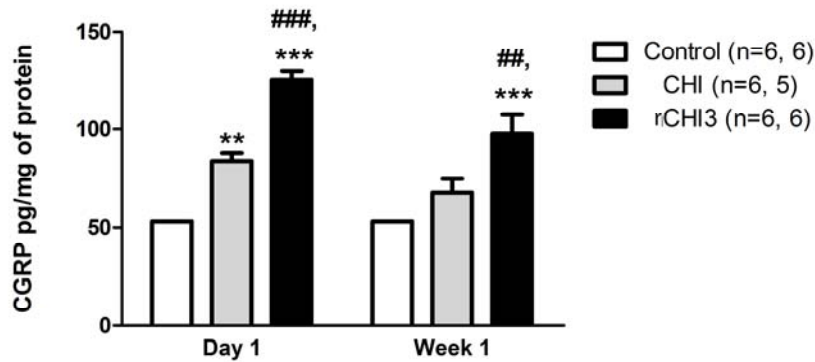


Figure 3: CGRP (pg/mg of protein) measured using an enzyme-linked immunosorbant assay to assess changes in the caudal brainstem at 1 day and 1 week endpoints post injury. The effects of single and rCHI on CGRP levels in the trigeminal nucleus caudalis (n=6 at day 1 and n=5 at week 1 endpoint) and rCHI groups (n= 6/endpoint), **p<0.01 and ***p<0.001 compared to incision control (n=6), and ##p<0.01 and ###p<0.001 compared to CHI.

We found evidence of a change in microglial number and phenotype in the central trigeminal pain system (TNC) (**Fig. 4**) in the presence of CGRP increase and absence of axonal injury which may contribute to a hyper-excitatory neuronal environment promoting a pain phenotype in our models . To test this, we examined the TNC for evidence of gliosis and found increases in

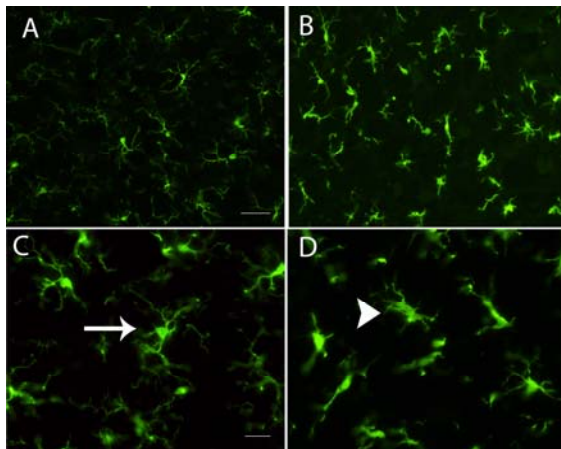


Figure 4: Microglia labeled with Iba-1 is shown for the trigeminal nucleus caudalis ophthalmic V1 region for control (A,C) and repetitive mild closed head injured rats (B,D). Microglia with thinly ramified processes (arrow; C) and retracted and thickened processes are observed in rCHI rats (arrowhead; D).

GFAP immunoreactivity in rCHI groups that was not present in controls (**Fig. 5**). in the TNC excitatory mechanisms as evidenced by increased CGRP and astrocytosis appear to contribute to the generation of acute headache behavior after mild closed head injury in rats. Astrocytosis was also found in the sensory cortex early (1 week) after repetitive mild closed head injury in rats (**Figure 6**). **However, in our project, the CB2 receptor is found on microglia in which**

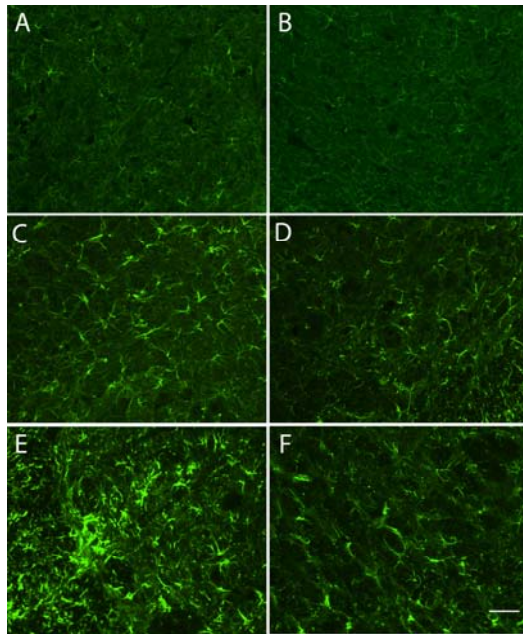


Figure 5: GFAP immunoreactivity at week one after control or mild closed head injury (mCHI) in the trigeminal nucleus caudalis ophthalmic V1 region is shown for the right (A,C, E) and Left (B,D,F) sides. GFAP immunoreactivity for (A,B) Incision, (C,D) single mCHI, and (E,F) repetitive mCHI₃. Scale bar = 50 μ m

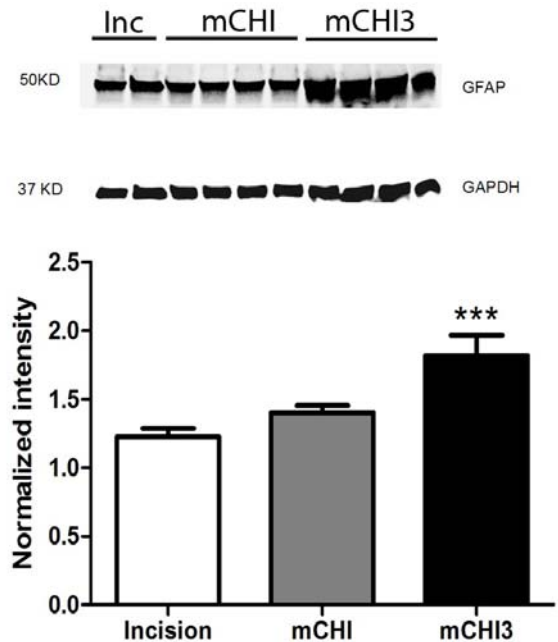


Figure 6: Western blot GFAP expression (D). GFAP expression quantification for the ipsilateral somatosensory cortex of incision, single closed head injured (mCHI; n=4), and repeated mCHI₃ (n=6) rats compared to incision n=4, $p < 0.001$ (E).

case the effects in the TNC is expected to be directly on microglia, but may also indirectly affect astrogliosis and neuronal activity. In our 3rd aim, we will examine this hypothesis in CB2 treated animals. In summary, our acute results show proinflammatory mechanisms contribute to excitatory changes in the TNC shown as increases in CGRP and altered microglial activity and increases in iNOS signaling, as well as astrogliosis most likely due to excess glutamate and/or CGRP release. The thalamic pain regions were negative for inflammatory markers except for the reticular thalamic nucleus showing iNOS signaling (shown in previous reports) which is an area for further investigation. Prostaglandin levels were not significantly increased in the thalamus, or TNC ($p=0.98$ and $p=0.31$, respectively).

Chronic group analysis:

Increased power after rCHI across several frequency bands in EEG recordings: We performed frequency analysis of EEG recordings in rCHI and control animals each week for four weeks post injury. In the first week post-injury, power across the spectrum of frequencies analyzed was similar between rCHI and control animals. However, power significantly increased over time in rCHI animals but not control animals (see Fig 8). The increase in power was

observed during all three periods examined: Pre-photostimulation, during photostimulation, and post-photostimulation.

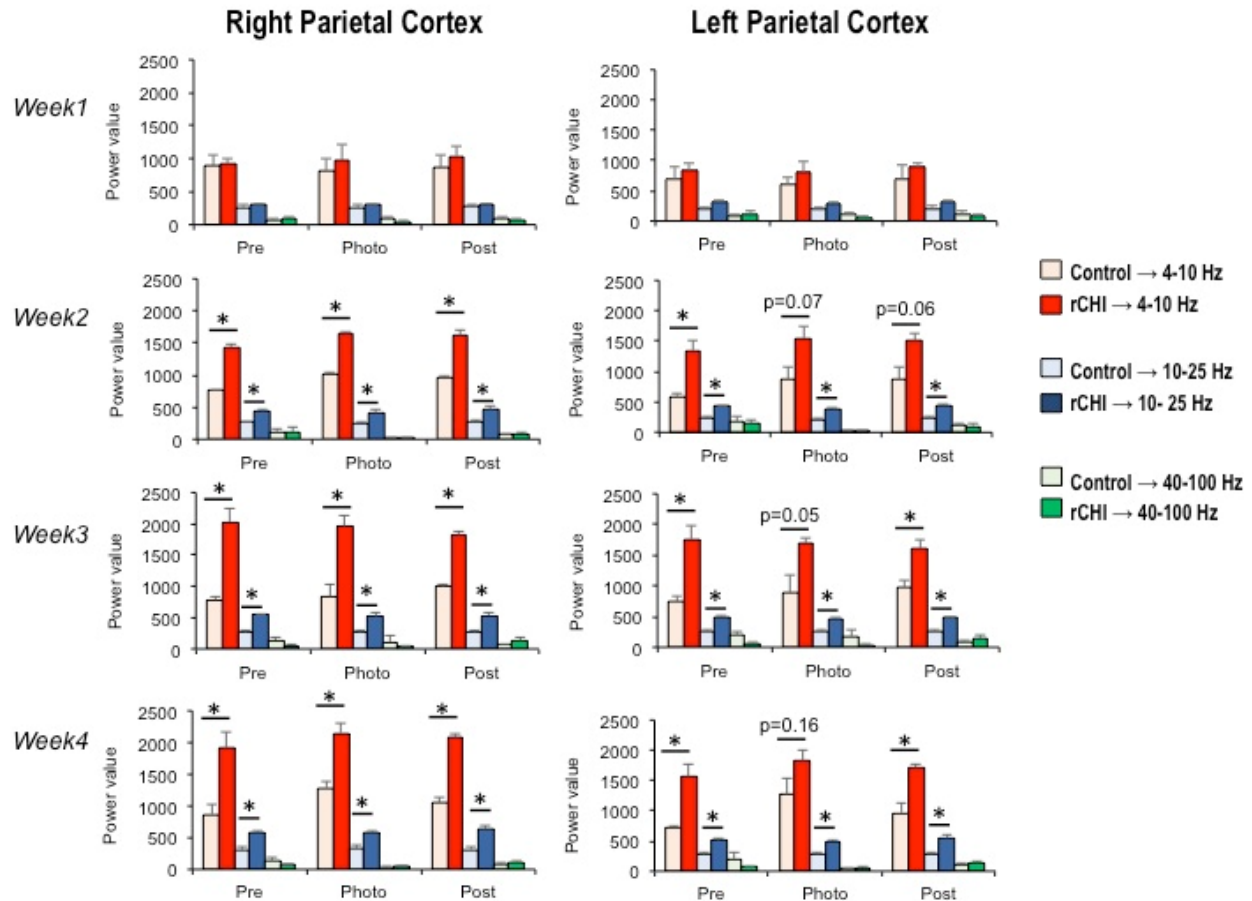


Figure 8. During the four weeks post-injury, rCHI animals develop an increase in power over several frequency bands compared to controls. Power is illustrated in three frequency bands: 4-10 Hz, 10-25 Hz, and 40-100 Hz (see legend). Data from left and right parietal cortex of rCHI animals is shown across the four weeks post-injury. Whereas power in all frequency bands remains relatively constant over time in control animals, the power increases over time for rCHI animals. “Pre”, Pre-photostimulation; “Photo”, during photostimulation; “Post”, Post-photostimulation.

We also performed similar frequency analysis of EEG recordings in sCHI and control animals each week for four weeks post injury. In contrast to rCHI animals, we found that there was no difference in power across any of the three frequency bands analyzed between sCHI and control animals, at any point over the course of the four weeks post injury (see Fig 8). These data demonstrate that EEG recordings reveal quantitative differences in brain activity between sCHI and rCHI models.

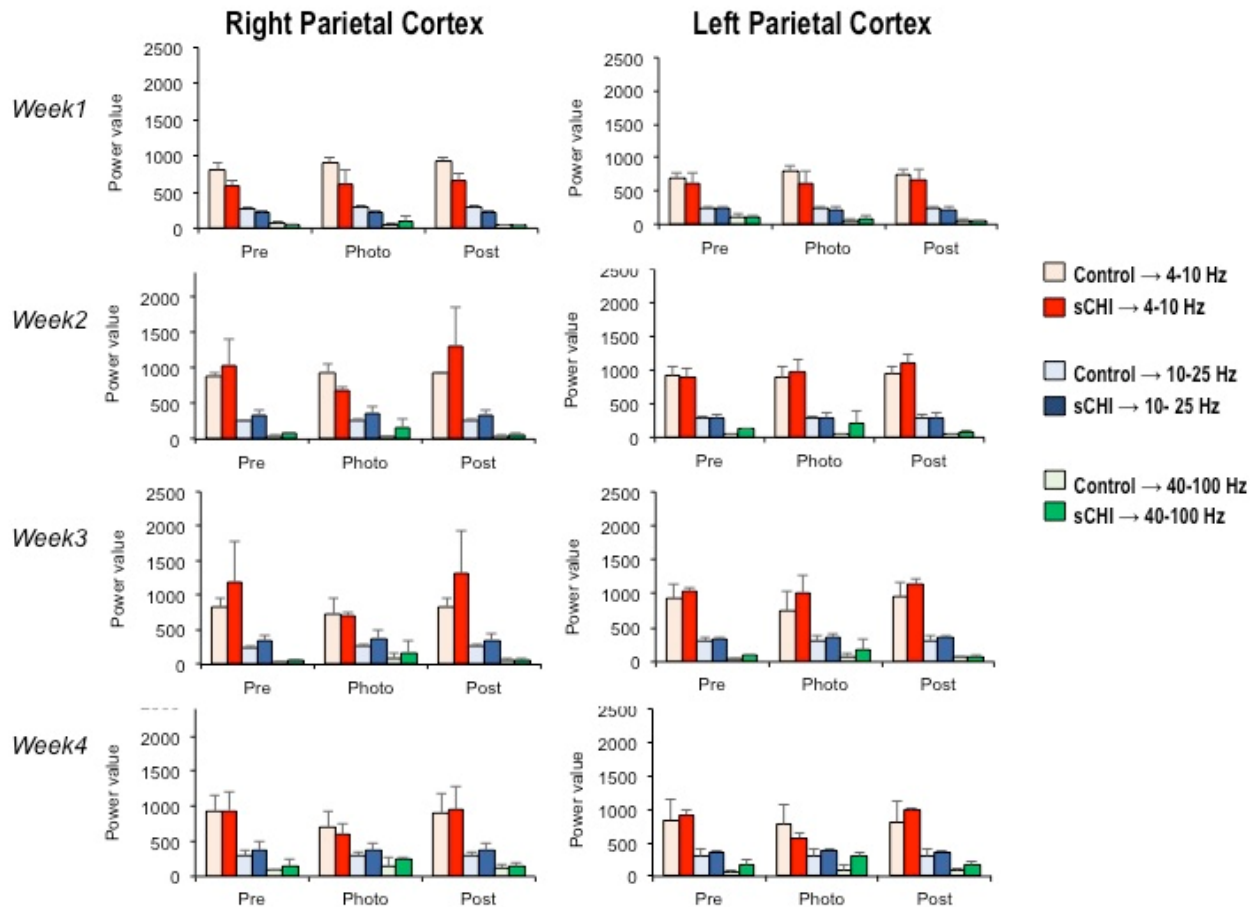


Figure 9. EEG power over several frequency bands is stable over time in sCHI animals, similar to control animals. Power is illustrated in three frequency bands: 4-10 Hz, 10-25 Hz, and 40-100 Hz (see legend). Data from left and right parietal cortex of rCHI animals is shown across the four weeks post-injury. Whereas power in all frequency bands remains relatively constant over time in control animals, the power increases over time for rCHI animals. “Pre”, Pre-photostimulation; “Photo”, during photostimulation; “Post”, Post-photostimulation.

EEG patterns in rCHI rats that are sensitive to photostimulation: During the period post-injury, rCHI animals develop sensitivity to the effects of photostimulation that outlast the actual photostimulation, as illustrated below. We created heat maps to illustrate the EEG power at each given frequency. At Week 1 post-injury, rCHI animals exhibit EEG activity that is very similar to controls, (similarity to controls is shown in “green”, an increase in “black-red” would indicate an increase in power, see Fig 9). However, by Week 4, rCHI animals develop not only the increase in the predicted frequency analysis (in nearly all frequency bands), they also develop sensitivity to the photostimulation as defined by a robust increase in EEG power at certain frequencies in the period following photostimulation,

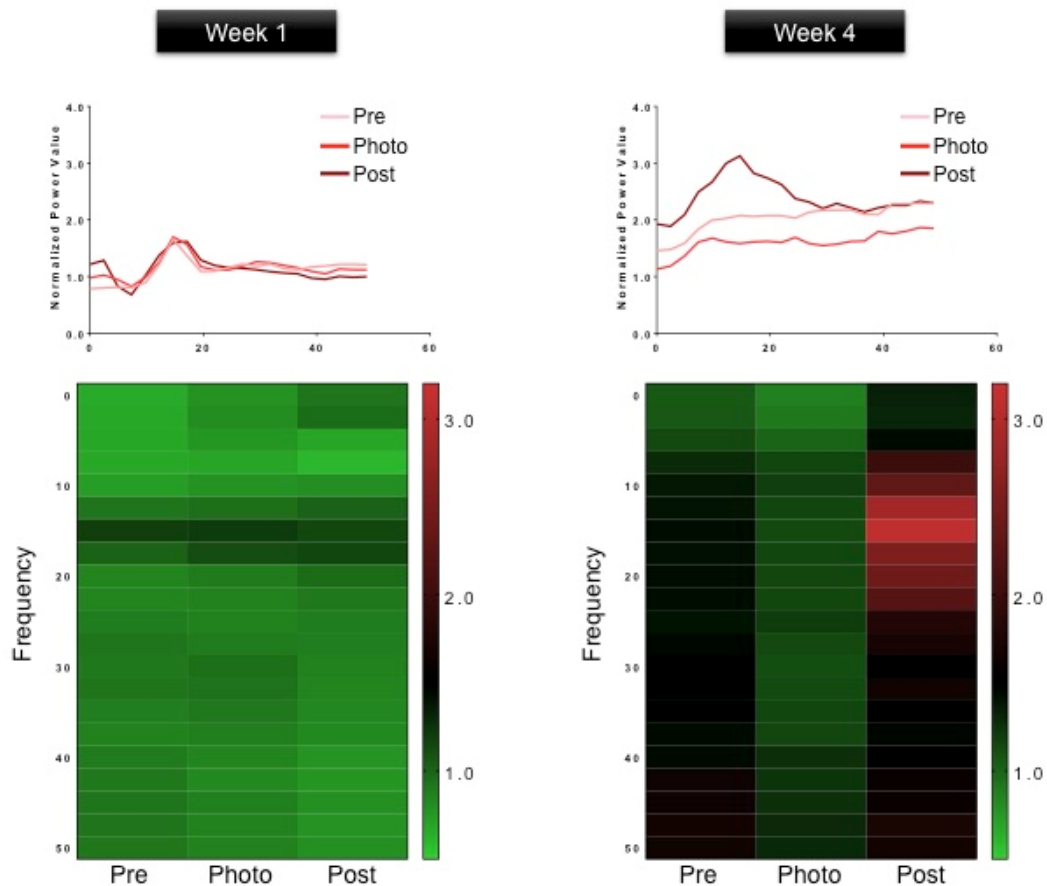


Figure 10. rCHI animals develop sensitivity to photostimulation that outlasts the actual photostimulation. Data from right parietal cortex of rCHI animals is shown at one- and four-weeks post-injury. At Week 1, power across EEG frequencies is similar between control and rCHI animals, however, by Week 4, EEG power is generally higher in rCHI animals, particularly in the period immediately photostimulation. “Pre”, Pre-photostimulation; “Photo”, during photostimulation; “Post”, Post-photostimulation.

Specific EEG signatures are exhibited for sCHI and rCHI rats: In addition to alterations in EEG power, we found that high amplitude oscillatory bursts developed over the course of the four weeks post injury in rCHI animals (see Fig 10). Although such bursts were not present the first week after injury, they began to occur by two weeks post injury, and were frequent by four weeks post injury. In contrast, we found that sCHI animals did not exhibit these high amplitude oscillatory bursts. However, they did exhibit very brief, periodic bursts of activity that were qualitatively distinct from those observed in rCHI animals (see Fig 10). These results demonstrate that the EEG activity in rCHI and sCHI animals is both qualitatively and

quantitatively distinct, and reliably distinguishes between rCHI and sCHI animals. Both types of bursts may provide useful biomarkers of activity that can be used to evaluate potential therapeutics.

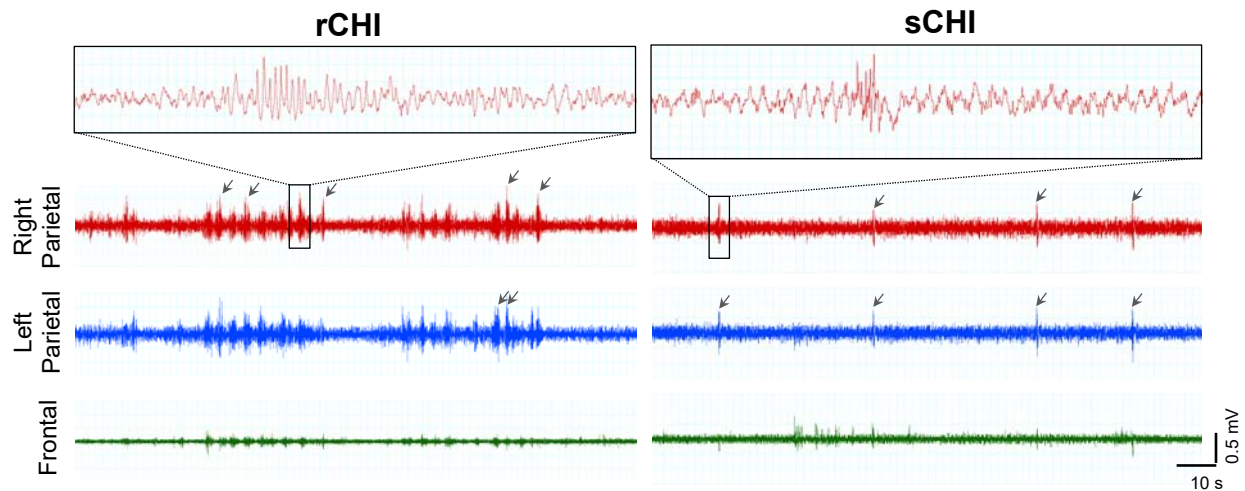


Figure 10. Robust high amplitude oscillatory bursts occur in rCHI but not sCHI animals. High amplitude oscillatory bursts develop over time after injury in rCHI animals (left panels, see arrows) – some are observed by two weeks after injury but are frequent by four weeks post injury. In contrast, sCHI animals develop distinct periodic brief bouts of high amplitude activity (right panels, see arrows) after four weeks post injury.

The high amplitude bursts in rCHI animals can be characterized using power spectral density (PSD) plots as seen in Fig 5. At week 1 post-injury, there is negligible difference between control and rCHI animals. However, by Week 4 after the injury, PSDs reveal the presence of a second peak or “shoulder” in rCHI animals that likely correspond to the frequency of the high amplitude bursts these mice exhibit (**as Fig 11**).

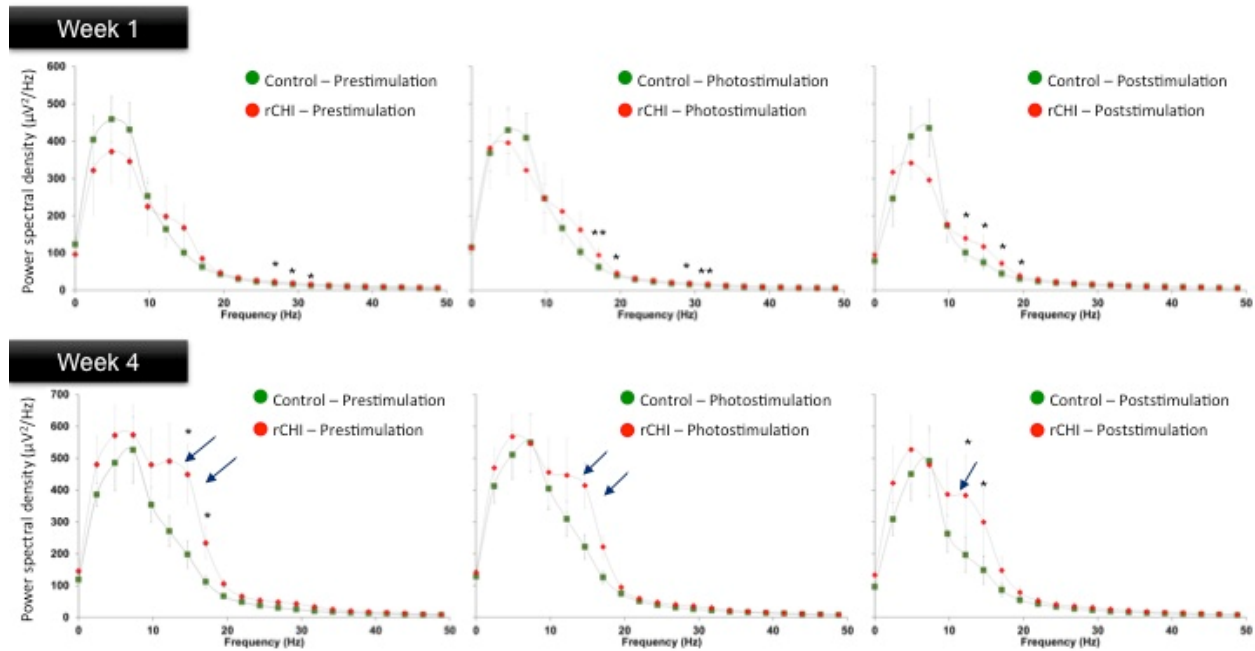


Figure 11. Power spectral density plots reveal a second peak of dominant EEG activity in rCHI animals that develops over time.

Quantitative EEG analysis and comparison of control animals with animals receiving single (sCHI) or repeated closed head injury (rCHI) has allowed us to identify several measures that reliably distinguish rCHI animals from sCHI or control animals, as described above. Our previous analysis of the chronic marker of neuronal activation, deltaFosB protein levels in our chronic cohorts showed a decrease in sensory cortex after rCHI compared to controls (**Figure 12**). This data was intriguing and is complex when coming with our qEEG data which show an increase in power. We propose that the reduction in chronic neuronal activation may be reflective of the basal inhibitory circuit in which case the net result would be hyper-activity in the cortex.

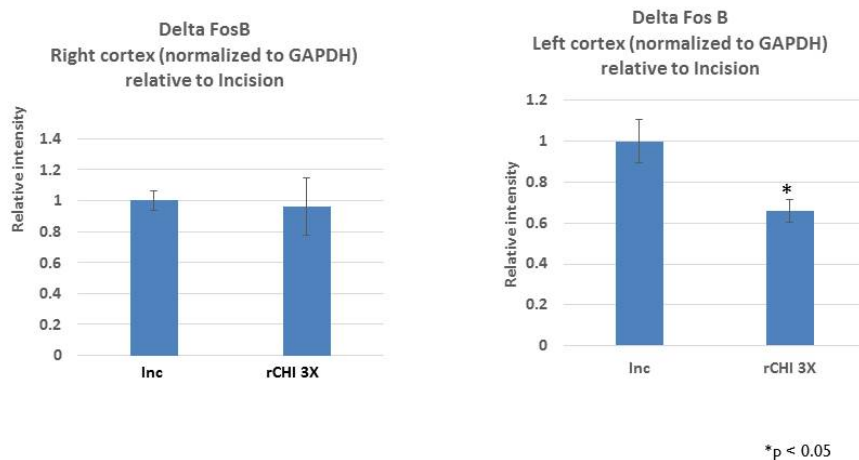


Figure 12: Delta Fos B protein quantified using western blot in the right and left cortex for incision control and rCHI3. Protein expression relative to incision controls (4/group) *p<0.05.

- **What opportunities for training and professional development has the project provided?** *Nothing to Report*
- **How were the results disseminated to communities of interest?** *Nothing to Report*
- **What do you plan to do during the next reporting period to accomplish the goals?**
 - Complete slice experiments for remaining chronic groups.
 - Complete remaining analysis of chronic tissues.
 - Submit manuscript on acute findings for publication.
 - Prepare manuscript on chronic EEG findings.
 - Begin aim 3 drug testing.

- **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
- Our findings have enhanced our understanding of the mechanisms underlying post-traumatic headache. In addition, the use of non-invasive EEG combined with light stimuli in patients with post-traumatic migraine is novel. Once published, results have the potential to directly impact the clinic in this population.
- **What was the impact on other disciplines?**
- Nothing to Report
- **What was the impact on technology transfer?** *Nothing to Report*
- **What was the impact on society beyond science and technology?**

Nothing to Report

- **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**
- Nothing to Report*
- **Actual or anticipated problems or delays and actions or plans to resolve them**
- *Nothing to report*
-
- **Changes that had a significant impact on expenditures**
- Nothing to report*
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
- Nothing to report*
- **Significant changes in use or care of human subjects:**
- Nothing to Report*

- **Significant changes in use or care of vertebrate animals:**

Nothing to Report

- **Significant changes in use of biohazards and/or select agents:**

Nothing to Report

- **PRODUCTS:**

- **Publications, conference papers, and presentations**

Journal publications.

- **Books or other non-periodical, one-time publications.**

- **Other publications, conference papers, and presentations.**

Invited Speaker Presentation: Title: The Role of the Cannabinoid Receptor Type-2 in Head Trauma: Studies on Inflammation and Pain Mid-Atlantic Pharmacology Society, Thursday, October 22, 2015, Cooper Medical School Rowan University, Camden, NJ

Invited Speaker Presentation Title: Cannabinoid Type-2 receptors modulate trigeminal pain signaling molecules and allodynia in a model of post-concussion headache, Carolina Cannabinoid Consortium Symposium, Double Tree, Philadelphia Center City, Sunday October 30, 2016

- **Website(s) or other Internet site(s)**

<http://www.jefferson.edu/university/jmc/departments/neurosurgery/faculty/elliott.html>

- **Technologies or techniques** *Nothing to Report*

- **Inventions, patent applications, and/or licenses** *Nothing to Report*

- **Other Products:**

Biospecimen collections were generated for a portion of the acute and chronic study groups for concussion model and model of post-traumatic headache.

- **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	Melanie Elliott	Jeannie Chin	Jarred Stratton	Ashley Tyburski	Mark Pyfer	Lan Cheng
Project Role:	PI	Co-I	Graduate Student	Research Technician	Research Technician	Research Associate
Researcher ID:	n/a	n/a	n/a	n/a	n/a	n/a
Month worked:	2.5	1.0	6	6.0	1.2	.25
Contribution:	Experimental design, data interpretation and presentation	EEG Setup, Experiments Analysis	Behavior, animal care, immunohistochemistry	Behavior, animal care, immunohistochemistry	EEG setup, surgery, experiments	EEG surgery, PCR

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?** JEANNIE

Nothing to Report

- **What other organizations were involved as partners?**

Nothing to Report

- **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:** N/A

- **QUAD CHARTS:** Attached

- **APPENDICES:** Nothing to report

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Central pain mechanisms and novel therapeutic strategies in a model of closed head injury



PI: Melanie Elliott, PhD

Org: Thomas Jefferson University

Award Amount: \$1,446,781.80

Study/Product Aim(s)

- **Specific Aim 1:** To identify the pattern of inflammation-induced sensitization of the central trigeminal pain neurons, and if sensitization is detectable by quantitative EEG.
- **Specific Aim 2:** To determine if anti-inflammatory mechanisms inhibit chronic sensitization of central trigeminal pain neurons in vitro.
- **Specific Aim 3:** To assess the in vivo therapeutic efficacy of a novel, non-psychoactive cannabinoid agent in a model of post-traumatic headache.

Approach: (1) Single/repeated mild closed head injury (CHI) will be induced in rats. Markers of inflammation and neuronal activation will be assessed in pain regions. Sensory testing will be compared to pain markers. EEG will be performed during exposure to a variety of sensory stimuli. (2) In vitro brain slices from injured or control groups will be bathed in inflammatory solutions, with or without anti-inflammatories, and the supernatant and tissues analyzed. (3) In vivo drug testing for a novel anti-inflammatory agent will be performed in CHI groups and compared to vehicle-treated groups.

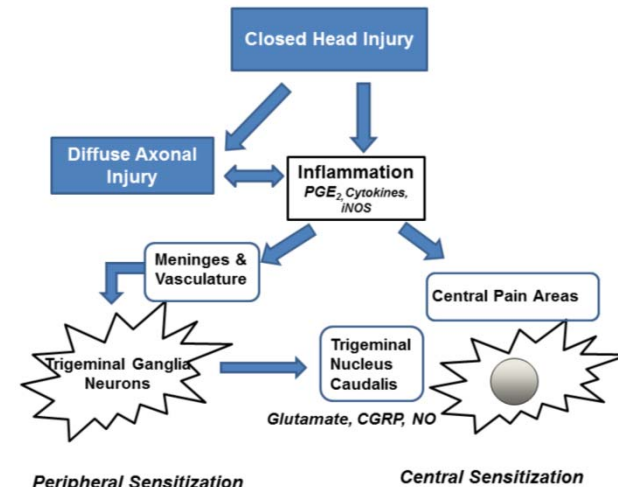


Figure: Proposed mechanisms of post-traumatic sensitization of the trigeminal pain circuit. Prostaglandins (PGE₂), inducible nitric oxide synthase (iNOS), calcitonin gene-related peptide (CGRP),

Timeline and Cost

Activities	CY	14	15	16	17
Aim 1					
Aim 2					
Aim 3					
Estimated Budget (\$K)		\$552K	\$443K	\$451K	

Updated: (October 28, 2016)

Goals/Milestones

CY14-15 Goal – Pain mechanisms and diagnosis

- ☐ Determine altered inflammatory and neuronal markers in the pain pathway in a model of CHI.
- ☐ Develop quantitative EEG to assess sensory changes in a model of CHI.

CY15-16 Goals –Anti-inflammatory strategies in vitro

- ☐ Determine the role of inflammatory stimuli in chronic neuronal sensitization implicated in pain.
- ☐ Determine the best anti-inflammatory strategy to minimize neuronal sensitization implicated in pain.

CY16-17 Goal – In vivo novel anti-inflammatory treatment efficacy

- ☐ Test novel and classical anti-inflammatories in an in vivo model of closed head injury